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In vivo reduction or blockade of interleukin-1β in primary osteoarthritis influences expression of mediators implicated in pathogenesis

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SUMMARY

Objective: Diminish interleukin-1β (IL-1β) signaling in a model of primary osteoarthritis by RNA interference-based transcript reduction or receptor blockade, and quantify changes incurred on transcript expression of additional mediators.

Methods: Knees of Hartley guinea pigs were collected at 120 and 180 days of age following injection with viral vectors ($N = 4$/treatment group/date) at 60 days. Two groups received either adeno-associated viral serotype 5 vector containing a knockdown sequence (TV), or adenoviral vector encoding for IL-1 receptor antagonist protein (Ad-IRAP); treatments were contrasted with opposite knees administered corresponding vector controls. A third group evaluated TV relative to saline-only injected knees. Chondropathy and immunohistochemistry findings were compared to untreated guinea pigs. Transcript expression levels in cartilage were calculated using the comparative CT ($2^{-\Delta\Delta CT}$) method and analyzed by one-way analysis of variance (ANOVA) with pairwise comparisons using Tukey 95% confidence intervals.

Results: Vector transduction was confirmed at both harvest dates. TV and Ad-IRAP, relative to vector controls, significantly decreased IL-1β. Inflammatory mediators [tumor necrosis factor-α (TNF-α), IL-8, interferon-γ (IFN-γ)], and catabolic matrix metalloproteinase 13 (MMP13) were also decreased, while anabolic transforming growth factor-β1 (TGF-β1) was increased. IL-1β was also decreased by TV vs saline, with a decrease in MMP13 and increase TGF-β1; TNF-α, IL-8, and IFN-γ were transiently increased.

Conclusions: This work confirmed that a reduction in IL-1β signaling was accomplished by either method, resulting in decreased expression of three inflammatory mediators and one catabolic agent, and increased expression of an anabolic molecule. Thus, evidence is provided that IL-1β serves a role *in vivo* in spontaneous osteoarthritis and that these translational tools may provide beneficial disease modification.

Introduction

Osteoarthritis (OA) is the leading cause of physical disability in developed nations[1]. Unfortunately, multiple molecular and biomechanical factors contribute to the pathogenesis of this degenerative process and restorative treatment options continue to be elusive[2]. Interleukin-1β (IL-1β), a pro-inflammatory cytokine that stimulates joint tissue to produce several proteases involved in cartilage degradation, is implicated as a principal instigator of OA[3]. Inhibiting the biological activities of IL-1β through RNA interference (RNAi) or receptor antagonism may delineate the contribution of its signaling pathway to disease and offers promise as a translational therapeutic strategy. Efforts to reduce or block the effective concentration of IL-1β have been demonstrated using diacerein, a compound that inhibits IL-1β production from synovium and cartilage[4–10], or IL-1 receptor antagonist protein (IRAP) in experimental OA[11–15]. Although these studies suggest that IL-1β is a viable target to modify development and succession of secondary joint deterioration, the majority of human cases of OA are idiopathic[16] and work is warranted to evaluate the definitive role of IL-1β in the context of spontaneous disease[3,17].

Recently, our laboratory provided a comprehensive immunohistochemical map describing the temporal expression and tissue distribution of IL-1β through progression of OA in two strains of guinea pigs with varying propensity for spontaneous knee joint disease[18]. At 60 days of age, IL-1β was detected in cartilage, menisci, synovium, and subchondral bone in both strains. Persistent

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expression was found in these tissues in OA-prone Hartley animals at 120 and 180 days, while OA-resistant Strain 13 animals demonstrated a significant reduction in immunostaining. Enduring IL-1β levels in Hartley animals appeared to coincide with histologic onset of OA, and this aberrant expression may correlate to early incidence of disease. Although findings suggested, but did not confirm, a cause-and-effect relationship between IL-1β expression and OA, we demonstrated a window when targeted cytokine reduction and/or blockade may identify such a connection and point toward mechanistic components. As such, the primary aim of this study was to minimize IL-1β-mediated signaling *via* RNAi-based transcript reduction or receptor blockade and quantify changes incurred on expression of mediators implicated in pathogenesis. We accomplished these aims *via* independent administration of a validated adeno-associated viral serotype 5 vector (AAV5) containing a short hairpin (sh)RNA knockdown sequence[19], or an adenoviral (Ad) vector encoding for recombinant human IRAP (hIRAP). These treatments were contrasted with opposite knees receiving corresponding vector controls with the goal of defining the contribution of IL-1β to spontaneous OA. A secondary aim was to challenge the efficacy and potential therapeutic viability of the AAV5 vector relative to saline-only injected control knees. Histologic chondropathy and immunohistochemistry (IHC) findings were compared to untreated guinea pigs.

Materials & methods

This study was performed according to the Guide for the Care and Use of Laboratory Animals of the NIH; all procedures were approved by the Institutional Laboratory Animal Care and Use Committee at the host university. Male Hartley guinea pigs (Charles River Laboratories, Wilmington, MA) were utilized in this study ($N = 32$) starting at 60 days of age. Animals were housed individually in solid bottom cages and allowed *ad libitum* water and guinea pig chow (Harlan Teklad 7006) until euthanasia. Body weight (grams) at time of harvest was recorded.

Experimental design

AAV5 vectors allowed simultaneous expression of shRNA *via* the U6 promoter and cytomegalovirus (CMV)-driven enhanced green fluorescent protein (GFP) expression. Plasmids containing the IL-1β specific shRNA knockdown sequence or non-targeting shRNA control sequence (Table IA) were used to produce targeting knockdown vector (TV) and non-targeting control vector (NTV), respectively, by the Viral Vector Core at Nationwide Children's Hospital[19,20]. Ad vectors contained coding regions for CMV-driven firefly luciferase (Ad-Luc) or hIRAP (Ad-hIRAP) and were propagated, as described[12]. Active protein production from Ad vectors was confirmed *in vitro* using bioluminescent luciferase detection or ELISA analyses for hIRAP (R&D Systems, Minneapolis, MN); results were positive for intended proteins, only.

Animals were injected at 60 days of age (four treatment groups; four animals/treatment group per collection date) and harvested at either 120 or 180 days of age. Vectors were aseptically administered into the medial aspect of the knee just distal to the femoral condyle using a 1/2cc 28g insulin syringe (Becton Dickinson, Franklin Lakes, NJ). Animals were assigned to one of two groups to investigate reduction/blockade of IL-1β vs respective vector controls: TV or NTV (1×10^{12} DNase resistant particles) were injected into opposite knees (group 1); Ad-Luc or Ad-hIRAP (2×10^{11} infectious units (IFUs)) were injected into opposite knees (group 2). Group 3 animals received TV or an equivalent volume of phosphate buffered saline (PBS) in opposite knees to challenge the efficacy of TV in the absence of vector control. Group 4 animals did not receive injections in either knee to allow reference of the above treatment groups to untreated controls. A total volume of 100 μl was administered; dosages and final injection volume were consistent with peer-reviewed manuscripts[19,20].

For groups 1–3, RNA was extracted from weight-bearing cartilage taken from the lateral femoral condyle and lateral tibial plateau of opposite knees for gene expression analyses. DNA was collected from patellar cartilage to quantify viral particle numbers. The medial femoral condyle and medial tibial plateau were processed for IHC and histology. Whole knee joints from group 4 animals were processed for IHC and histology.

Table I
A. Targeting knockdown and control shRNA sequences

Targeting knockdown IL-1β sequence	GCCAGGATATAATTGACTTCACGAATGAAGTCAATTATATCCTGGC
Non-targeting control sequence	GGATATATCCCGAACTAGACACGAATGTCTAGTTCGGGATATATCC

B. Specific primers used for real-time qRT-PCR

Transcript of interest	Primer sequences (5′–3′)
18S ribosomal RNA	F: TGCATGGCCGTTCTTAGTTG
	R: AGTTAGCATGCCAGAGTCTCGTT
GAPDH	F: GTATCGTGGAAGGACTCATGACC
	R: GTTGAAGTCACAGGACACAACCT
GFP	F: CATGATATAGACGTTGTGGCTGTTG
	R: AAGCTGACCCTGAAGTTCATCTGC
Firefly luciferase*	F: GCCTGAAGTCTCTGATTAAGT
	R: ACACCTGCGTCGAAGT
CVM promoter	F: GGTCTATATAAGCAGAGCTG
	R: GTGGTATGGCTGATTATGATCAG
IL-1β	F: ACGCCTGGTGTTGTCTGAC
	R: GGGAACTGAGCGGATTC
IL-8	F: GGCAGCCTTCCTGCTCTCT
	R: CAGCTCCGAGACCAACTTTGT
Human IRAP†	F: TGGCTAACTAGAGAACCCACTGCT
	R: TTCTGAAGGCTTGCATCTTGCTGG
Collagenase 3 (MMP13)	F: TTCTGGCACATGCTTTTCCTC
	R: GGTTGGGGTCTTCATCTCCTG
TGF-β1	F: CATCGATATGGAGCTGGTGAAG
	R: GCCGTAATTTGGACAGGATCTG
TNF-α	F: CCTACCTGCTTCTCACCCATACC
	R: TTGATGGCAGAGAGAAGGTTGA
INF-γ	F: ATTTCGGTCAATGACGAGCAT
	R: GTTTCCTCTGGTTCGGTGACA

* Fan X, Roy E, Zhu L, Murphy TC, Kozlowski M, Nanes MS, Rubin J. Nitric oxide donors inhibit luciferase expression in a promoter-independent fashion. J Biol Chem. 2003; 278(12):10232–8.

† Jia X, Cheng K, Mahato RI. Co-expression of vascular endothelial growth factor and IL-1 receptor antagonist for improved human islet survival and function. Mol Pharm. 2007; 4(2):199–207.

Quantification of CMV copy number

DNA was isolated *via* the QIAamp® DNA Mini kit (Qiagen, Valencia, CA) according to manufacturer's protocol. DNA quality and concentration were determined using the BioMate 3 spectrophotometer (Thermo Fisher Scientific, Waltham, MA). Absolute quantification of initial CMV copy number per 1 μg starting DNA was performed in triplicate using SYBR® Green PCR Master Mix (Applied Biosystems, Warrington, UK) and primers specific for the CVM promoter (Table IB) on the ABI Prism® 7000 Sequence Detection System (Applied Biosystems, Foster City, CA). A standard curve ranging from 10^7 to 10^1 starting particles was prepared using the TV plasmid. If amplification was not detected, negative

results were verified by repeating reactions with up to 2 μg starting DNA.

Relative real-time (Q)RT-PCR

Total RNA was isolated *via* TRIzol® (Invitrogen, Carlsbad, CA), followed by DNase I treatment (Invitrogen, Carlsbad, CA), according to manufacturer's protocol. Complementary DNA was made using Taqman® Reverse Transcription Reagents (Applied Biosystems, Foster City, CA) and relative qRT-PCR, complete with dissociation curve, was performed in triplicate for each transcript of interest using Power SYBR® Green PCR Master Mix (Applied Biosystems, Warrington, UK) and specific primers (Table IB) on the ABI Prism® 7000. 18s ribosomal RNA and Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) were used as separate endogenous controls to which each transcript was normalized. Results were consistent between housekeeping genes; ΔCT values using GAPDH are represented. If amplification was not detected, reactions were repeated using a higher starting amount of cDNA to confirm negative findings. ΔCT values for GFP transcripts in groups 1 and 3 at 120 and 180 days were reported. Fold differences and subsequent percent gene expression levels relative to designated control groups were calculated for each transcript using the comparative cycle threshold (CT) ($2^{-\Delta\Delta CT}$) method. For all groups, comparative CT values for each treatment group were compared to opposite control knees.

Luciferase expression

Immediately prior to euthanasia, *in vivo* bioluminescent imaging was performed to ensure luciferase activity in knees receiving Ad-Luc. Group 2 animals at 120 and 180 days were induced and maintained under general anesthesia using 2% isoflurane and received an intra-peritoneal injection of D-luciferin (Caliper Life Sciences, Hopkinton, MA) at a dose of 150 mg/kg. Fifteen minutes post-injection, animals were placed in a cooled charge-coupled device camera system (IVIS Imaging System-100; Xenogen/Caliper Life Sciences, Alameda, CA) for data acquisition according to recommendations[21].

Joint processing & analyses

Knee joints were fixed in 10% neutral buffered formalin and prepared for histological analysis[18,22–25]. Paraffin sections (5 μm) were taken from the center of the medial tibial plateau in each joint and either stained with toluidine blue or subjected to IHC. Four sections from each joint were examined using each scoring technique.

IHC was performed on the Benchmark XT (Ventana, Tucson, AZ) using the Ultraview Universal Red AP System and a rabbit polyclonal antibody for IL-1β (sc-7884; Santa Cruz Biotechnology, Santa Cruz, CA) at a dilution of 1:800. Sections were counterstained with hematoxylin. The percentage of chondrocytes staining positive for IL-1β were assessed by two independent, blinded reviewers (KSS/GN) and assigned a total IHC (based on the percentage and intensity of chondrocytes staining positive for IL-1β) ranging from *0–8*[18]. Intra- and inter-observer variability was negligible (within one numeric score in all cases).

Two independent, blinded observers (KSS/ALB) performed histological grading of serial coronal sections of each knee, using adapted Mankin criteria based upon species-specific features of OA[24,25]. Chondropathy was scored for the medial femorae and tibial plateaus, providing a total possible medial joint index ranging from *0–28*. Intra- and inter-observer variability was negligible (within one numeric score in all cases).

Statistical analyses

Individual samples harvested from each animal were considered independent observations; for each gene or transcript of interest, this resulted in four independent observations per treatment group per harvest date. ΔCT values for each gene or transcript of interest (triplicate values for each independent observation) passed Gaussian distribution and homogeneous variance using the Kolmogorov–Smirnov normality test. Statistical differences were not detected between animals within the same treatment group and harvest date. Weight, viral particle numbers, and relative percent expression qRT-PCR data (directly converted from fold changes) were expressed as mean ± 95% confidence interval and analyzed by one-way ANOVA followed by pairwise comparisons using Tukey 95% confidence intervals. Total indices for OA and IHC (median and range provided) were analyzed using the one-way ANOVA, Kruskal–Wallis test, followed by Dunn's Multiple Comparison post-hoc test[18]. All analyses were performed using the Minitab statistical software program (State College, PA) with a statistical significance of $P < 0.05$. Throughout the manuscript, indicated "increases" and "decreases" in transcript expression refer to significant statistical differences in presented data.

Results

Successful transduction of patellar cartilage by TV and NTV was confirmed *via* detectable amplification of the CMV promoter at 120 and 180 days [Fig. 1(A)]; significant statistical differences between days 120 and 180 or between groups 1 and 3 were not present. The CMV promoter was not detected in patellar cartilage harvested from group 2 animals injected with either Ad vector.

GFP expression was detected *via* qRT-PCR in knees receiving either TV or NTV. Significant statistical differences in ΔCT values were not present between days 120 and 180 or between groups 1 and 3 [Fig. 1(B)].

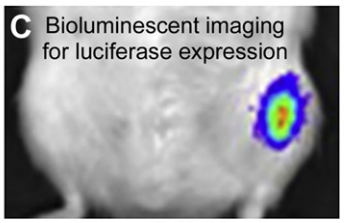
Luciferase expression in knees receiving Ad-Luc displayed positive bioluminescence immediately prior to collection, indicating successful transduction in joint tissue throughout the duration of the study [Fig. 1(C)]. Neither luciferase nor hIRAP transcripts was detected in cartilage *via* qRT-PCR, confirming that, along with lack of detection of CMV promoter, Ad-Luc and Ad-IRAP vectors transduced joint structures other than the examined cartilage.

IL-1β reduction/blockade vs respective vector controls

TV relative to NTV, and Ad-hIRAP relative to Ad-Luc, demonstrated decreases in IL-1β expression in cartilage at 120 days [Table II(A)]. IL-1β was below detection limits collected from both groups at 180 days. In general, TV and Ad-hIRAP decreased expression of tumor necrosis factor-α (TNF-α), IL-8, and interferon-γ (IFN-γ) relative to respective vector controls [Table II(B)]. TNF-α and IL-8 were decreased by TV compared to NTV at both harvest ages. Significant statistical differences in IFN-γ were not detected at 120 or 180 days. TNF-α was increased by Ad-hIRAP vs Ad-Luc at 120 days. This expression, however, was decreased relative to Ad-Luc by 180 days, revealing a significant statistical difference in Ad-hIRAP treated knees between 120 and 180 days (Supplemental Table I). IL-8 and IFN-γ were decreased by Ad-hIRAP at both collection times. Overall, anabolic transforming growth factor-β1 (TGF-β1) was increased by TV and Ad-hIRAP relative to vector controls at 120 days [Table II(C)]. This increase in group 1 continued at 180 days; however, TGF-β was decreased in group 2 at this time. Catabolic collagenase III/matrix metalloproteinase 13 (MMP13) was decreased by either method used to manipulate IL-1β at 120 days

A CVM copy number per 1μg DNA (mean ± 95% confidence intervals)

	Age at time of collection			
	120 days		180 days	
	Treated knee	Control knee	Treated knee	Control knee
Group 1 (TV/NTV)	7880 ± 1650	5680 ± 1822	6075 ± 1302	6182 ± 1405
Group 2 (Ad-hIRAP/Ad-Luc)	ND	ND	ND	ND
Group 3 (TV/PBS)	5326 ± 2063	ND	4987 ± 2232	ND



Group 2 (Ad-Luc)

B GFP transcript expression (mean ΔCT value ± 95% confidence intervals)

	Age at time of collection			
	120 days		180 days	
	Treated knee	Control knee	Treated knee	Control knee
Group 1 (TV/NTV)	7.18 ± 0.91	6.18 ± 0.99	6.71 ± 0.90	6.45 ± 0.97
Group 3 (TV/PBS)	8.04 ± 0.93	ND	6.95 ± 0.92	ND

Fig. 1. *In vivo* detection of the CMV promoter (A) in 1 μg starting DNA and expression of reporter genes for AAV5 (B) and adenoviral (Ad) vectors (C) were confirmed using PCR, qRT-PCR, and Xenogen IVIS System, respectively. DNA containing the CMV promoter was not detected (ND) in patellar cartilage collected from group 2 Ad-injected animals at either 120 or 180 days of age. A significant difference for this promoter between groups 1 and 3 was not present at either harvest date. Statistical differences in GFP expression were not noted in cartilage at 120 or 180 days of age in groups 1 and 3, as determined using one-way ANOVA followed by pairwise comparisons using Tukey's 95% confidence intervals. Luciferase (Luc) expression was detected in all Ad-Luc injected knees immediately prior to harvest on both collection ages (C). TV = AAV5-shRNA-TV; NTV = AAV5-shRNA-NTV; Ad-Luc, vector containing a CMV-driven coding region for firefly luciferase (Luc); Ad-hIRAP, vector containing a CMV-driven coding region for hIRAP.

[Table II(D)]. At 180 days, the decrease in MMP13 incited by TV relative to NTV persisted, while Ad-hIRAP increased expression relative to Ad-Luc.

Reduction of IL-1β by shRNA-TV relative to PBS control (group 3)

To stringently evaluate efficacy of novel TV in the context of primary OA, this vector was compared to opposite control knees receiving PBS, only (group 3). Successful *in vivo* transduction of patellar cartilage by TV was confirmed *via* detectable, and similar, amplification of the CMV promoter at both 120 and 180 days [Fig. 1(A)] and discernible, and comparable, ΔCT values for GFP between days 120 and 180 [Fig. 1(B)]. At 120 and 180 days, the IL-1β transcript was decreased relative to PBS control [Table III(A)]. At 120 days, TNF-α, IL-8, and IFN-γ were increased by TV relative to PBS [Table III(B)]. TNF-α and IL-8, however, were decreased by 180 days, while IFN-γ remained increased. TGF-β1 was increased by TV relative to PBS at 120 and 180 days, with impressive expression found at the latter time point [Table III(C)]. MMP13 was decreased by TV at both harvest dates [Table III(D)].

Comparison of Ad-Luc and Ad-hIRAP (group 2) to PBS control knees (group 3)

Comparison of Ad-Luc or Ad-hIRAP vs PBS was performed across animals. At 120 days, Ad-Luc-treated knees demonstrated increased IL-1β relative to PBS control knees [Table IV(A)]. IL-1β was below detection in Ad-hIRAP-treated knees at either time point; although detected at 120 days, IL-1β was not present in Ad-Luc treated knees at 180 days. All inflammatory cytokines of interest were increased by Ad-Luc and Ad-hIRAP relative to PBS control knees at 120 days [Table IV(B)]. TNF-α was increased compared to PBS exposed cartilage at 180 days and, in Ad-Luc

Table II
Relative percent expression (mean ± 95% confidence intervals) of (A) IL-1β, (B) inflammatory cytokines of interest, and (C) anabolic and (D) catabolic agents in cartilage of viral vector treated knees vs vector control knees (groups 1 and 2) at specified ages

		Group 1				Group 2		
		NTV	TV	*P* value		Ad-Luc	Ad-hIRAP	*P* value
A.	**IL-1β**							
	120 days	100.0 (84.1, 115.9)	5.5 (3.9, 7.1)	<0.001	120 days	100.0 (90.5, 109.5)	ND	<0.001
	180 days	ND	ND	NA	180 days	ND	ND	NA
B.	**TNF-α**							
	120 days	100.0 (80.9, 119.1)	15.3 (7.3, 23.3)	<0.001	120 days	100.0 (82.5, 117.5)	720.6 (584.1, 1,357.0)	<0.001
	180 days	100.0 (87.3, 112.7)	23.8 (7.9, 39.7)	<0.001	180 days	100.0 (85.7, 114.3)	33.0 (14.7, 80.7)	0.01
	IL-8							
	120 days	100.0 (84.1, 115.9)	ND	<0.001	120 days	100.0 (94.5, 105.4)	35.4 (15.5, 55.3)	<0.001
	180 days	100.0 (85.7, 114.3)	21.0 (5.1, 36.9)	<0.001	180 days	100.0 (83.7, 117.4)	32.2 (17.6, 51.9)	<0.001
	IFN-γ							
	120 days	100.0 (94.1, 105.9)	120.0 (65.8, 239.1)	>0.05	120 days	100.0 (90.4, 109.5)	5 (1.8, 8.2)	<0.001
	180 days	100.0 (86.8, 113.4)	75.0 (44.3, 194.3)	>0.05	180 days	100.0 (87.2, 113.7)	42.1 (18.2, 65.9)	<0.001
C.	**TGF-β1**							
	120 days	100.0 (92.0, 108.0)	643.0 (324.8, 961.2)	<0.001	120 days	100.0 (90.5, 109.5)	873.2 (236.7, 1,510.0)	<0.001
	180 days	100.0 (92.4, 108.0)	412.6 (223.9, 1,049.0)	<0.001	180 days	100.0 (84.1, 115.9)	32.5 (6.8, 64.3)	0.008
D.	**MMP13**							
	120 days	100.0 (94.1, 105.9)	ND	<0.001	120 days	100.0 (93.4, 107.5)	ND	<0.001
	180 days	100.0 (85.6, 114.3)	ND	<0.001	180 days	100.0 (87.3, 112.7)	200.0 (144.3, 255.7)	<0.001

ND = not detected; NA = not applicable.

Table III
Relative percent expression (mean ± 95% confidence intervals) of (A) IL-1β, (B) inflammatory cytokines of interest, and (C) anabolic and (D) catabolic agents in cartilage of AAV5 vector-exposed guinea pigs compared to PBS-treated knees (group 3) at specified ages

		Group 3		
		PBS	TV	*P* value
A.	**IL-1β**			
	120 days	100.0 (87.2, 112.7)	64.8 (45.7, 82.9)	0.03
	180 days	100.0 (88.8, 111.1)	42.0 (18.1, 65.8)	<0.001
B.	**TNF-α**			
	120 days	100.0 (93.6, 106.4)	266.0 (126.9, 425.1)	<0.001
	180 days	100.0 (92.0, 108.0)	25.0 (4.8, 64.7)	0.005
	IL-8			
	120 days	100.0 (87.2, 112.7)	179.0 (123.3, 234.7)	<0.001
	180 days	100.0 (88.6, 111.4)	70.0 (49.3, 90.7)	0.04
	IFN-γ			
	120 days	100.0 (82.7, 117.2)	608.0 (448.9, 767.1)	<0.001
	180 days	100.0 (90.6, 110.4)	1,087.0 (768.8, 1,405.0)	<0.001
C.	**TGF-β1**			
	120 days	100.0 (93.6, 106.4)	156.0 (116.4, 235.6)	0.009
	180 days	100.0 (92.0, 108.0)	542.7 (304.0, 781.4)	<0.001
D.	**MMP13**			
	120 days	100.0 (87.3, 112.7)	30.0 (16.1, 53.8)	0.003
	180 days	100.0 (88.6, 111.1)	10.0 (2.0, 17.9)	<0.001

treated knees, this expression was increased relative to the earlier time point [Table IV(B); Supplemental Table I]. IL-8 and IFN-γ were decreased at 180 days relative to 120 days within knees exposed to Ad vectors (Supplemental Table I). At 180 days, only Ad-Luc demonstrated an increase in IL-8 relative to PBS-exposed cartilage and both IL-8 and INF-γ were decreased in Ad-hIRAP treated cartilage relative to PBS knees by this harvest date. At 120 days, TGF-β1 was increased in cartilage treated with either Ad vector relative to PBS treated knees [Table IV(C)]. By 180 days, however, transcript expression was decreased in Ad-hIRAP treated knees. MMP13 was decreased by both Ad vectors relative to PBS control cartilage at 120 days, but increased at the latter harvest age [Table IV(D)].

IHC and histology

A final set of animals (group 4) did not receive injections in either knee to allow reference of IHC and chondropathy from treatment groups to untreated controls. IHC to detect IL-1β was performed and graded to corroborate qRT-PCR findings. Scores for IL-1β at the protein level reflected qRT-PCR data, where IHC indices were lower in groups 1, 2, and 3 relative to untreated group 4 at both 120 and 180 days [Fig. 2(A, B)]. Total OA indices for the medial joint compartments increased from 120 to 180 days, similar to that previously reported[18,22–25] [Fig. 2(B, C)]. Significant statistical differences in OA indices were not present among treatment groups within specified harvest dates. Significant statistical differences in weight were not detected among the treatment groups at either 120 or 180 days [Fig. 2(D)].

Discussion

The goals of our study were to demonstrate effective intra-articular vector transduction aimed at reducing IL-1β expression and/or signaling in OA-prone cartilage, quantify ensuing chondrogenic mediator profiles in articular cartilage, and provide compelling molecular evidence for potential disease modification. Both TV and Ad-hIRAP were successful at decreasing IL-1β expression in OA-prone cartilage at both the transcript and protein levels. Notably, this IL-1β diminution directly affected several key players implicated in joint degeneration. Relative to respective vector controls, these anti-IL-1β treatments reduced TNF-α, IL-8, IFN-γ, and simultaneously increased anabolic TGF-β and decreased catabolic MMP13[26,27]. Importantly, these same patterns were elucidated when TV was compared to contralateral PBS-exposed cartilage; these findings can be anticipated to allow a joint environment less acquiescent to pathology. As such, our work supplied confirmation that treatments aimed at decreasing the effective concentration of IL-1 and/or its signaling may hold merit for amendment of disease-prone cartilage.

To date, intra-articular hIL-1Ra injections to treat symptomatic knee OA in people found no statistical improvement over placebo at 1 month[28] and trials with diacerein did not show clinical, radiographic, or structure-modifying effects as anticipated[29–31]. Use of recombinant proteins and/or pharmaceutical compounds is limited not only by short half-lives and the requirement for repeat administration, but uncharacterized innate and adaptive responses to exogenous substances may also affect efficacy, including internalization or down-regulation of receptors. Although we demonstrated an ability to manipulate IL-1β signaling in cartilage such that correlative expression changes in other mediators could be

Table IV
Relative percent expression (mean ± 95% confidence intervals) of (A) IL-1β, (C) inflammatory cytokines of interest, and (D) anabolic and (E) catabolic agents in cartilage at specified ages in Ad vector treated animals vs PBS-treated knees

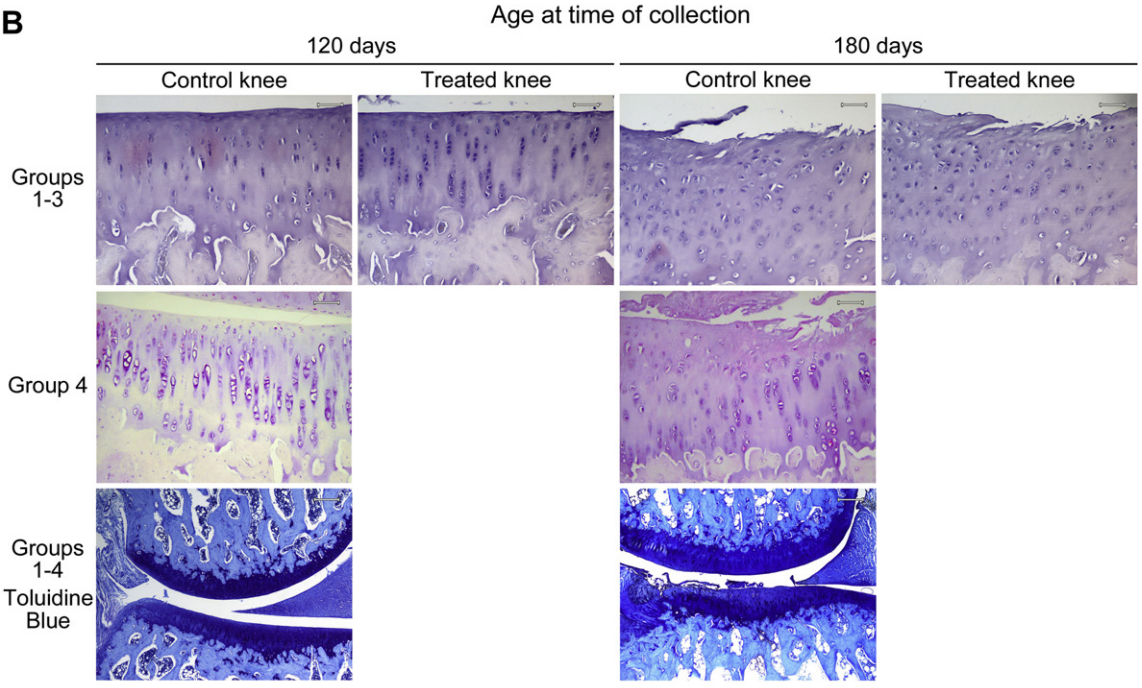
		PBS	Ad-Luc	*P* value		PBS	Ad-hIRAP	*P* value
A.	**IL-1β**							
	120 days	100.0 (87.2, 112.7)	362.0 (123.3, 600.7)	<0.001	120 days	100.0 (87.2, 112.7)	ND	<0.001
	180 days	100.0 (88.8, 111.1)	ND	<0.001	180 days	100.0 (88.8, 111.1)	ND	<0.001
B.	**TNF-α**							
	120 days	100.0 (93.6, 106.4)	1,420.0 (1,261.0, 1,579.0)	<0.001	120 days	100.0 (93.6, 106.4)	2,181.0 (1,544.0, 2,817.0)	<0.001
	180 days	100.0 (92.0, 108.0)	4,440.0 (1,258.0, 7,622.0)	<0.001	180 days	100.0 (92.0, 108.0)	1,465.0 (1,306.0, 1,624.0)	<0.001
	IL-8							
	120 days	100.0 (87.3, 113.1)	1,389.0 (672.9, 2,105.0)	<0.001	120 days	100.0 (87.3, 113.1)	492.0 (332.9, 651.1)	<0.001
	180 days	100.0 (88.8, 111.1)	173.0 (119.3, 252.6)	<0.009	180 days	100.0 (88.8, 111.1)	55.8 (23.9, 87.6)	0.04
	IFN-γ							
	120 days	100.0 (90.4, 109.5)	3,335.0 (1,744.0, 4,926.0)	<0.001	120 days	100.0 (90.4, 109.5)	166.8 (121.1, 222.4)	<0.001
	180 days	100.0 (87.7, 112.7)	84.0 (52.1, 115.8)	>0.05	180 days	100.0 (87.7, 112.7)	35.3 (19.4, 51.2)	0.006
C.	**TGF-β1**							
	120 days	100.0 (93.6, 106.3)	251.0 (127.8, 394.2)	<0.001	120 days	100.0 (93.6, 106.3)	2.1×10^5 (1.9×10^5, 2.5×10^5)	<0.001
	180 days	100.0 (84.1, 115.9)	222.0 (174.3, 269.7)	<0.001	180 days	100.0 (84.1, 115.9)	39.6 (25.3, 53.9)	0.01
D	**MMP13**							
	120 days	100.0 (87.3, 112.7)	50.0 (10.2, 79.8)	0.005	120 days	100.0 (87.3, 112.7)	ND	<0.001
	180 days	100.0 (89.6, 111.1)	179.0 (139.2, 218.8)	<0.001	180 days	100.0 (89.6, 111.1)	358.0 (198.9, 517.1)	<0.001

ND = not detected.

A Median (range provided) IL-1β IHC scores

	Age at time of collection			
	120 days		180 days	
	Treated knee	Control knee	Treated knee	Control knee
Group 1 (TV/NTV)	1 (0-2)	0 (0-1)	0 (0-2)	1 (0-2)
Group 2 (Ad-hIRAP/Ad-Luc)	0 (0-1)	0 (0-1)	1 (0-1)	1 (0-1)
Group 3 (TV/PBS)	0 (0-1)	1 (0-2)	0 (0-1)	1 (0-1)
Group 4 (Untreated)		6 (4-7)†		5 (4-7)†

B



C Median (range provided) OA scores

	Age at time of collection			
	120 days		180 days†	
	Treated knee	Control knee	Treated knee	Control knee
Group 1 (TV/NTV)	0 (0-2)	1 (0-2)	5 (4-10)	6 (4-11)
Group 3 (Ad-hIRAP/Ad-Luc)	1 (0-2)	2 (0-2)	5 (4-8)	7 (5-11)
Group 3 (TV/PBS)	2 (0-2)	2 (0-2)	6 (4-10)	7 (4-11)
Group 4 (Untreated)		0 (0-1)		6 (4-9)

D Mean (± 95% confidence intervalst) Body Weight (grams)

	Age at time of collection	
	120 days	180 days
Group 1 (TV/NTV)	745.5 ± 150.5	923.6 ± 172.0
Group 2 (Ad-hIRAP/Ad-Luc)	736.4 ± 118.2	911.8 ± 153.5
Group 3 (TV/PBS)	667.2 ± 30.4	901.7 ± 67.1
Group 4 (Untreated)	815.4 ± 272.8	903.6 ± 104.2

Fig. 2. Median (range provided) IHC scoring (A) and OA grading (C), mean body weight (D), and representative photomicrographs (B) (100, final magnification), of medial tibial plateaus from treatment groups harvested at specified ages. Animals were injected with viral vectors ($N = 4$ animals per treatment group per collection date) at 60 days of age and harvested at either 120 or 180 days of age. At time of treatment, animals were assigned to one of two groups to investigate reduction or blockade, respectively, of IL-1β vs respective vector controls: TV or NTV were injected into contralateral knees (group 1); Ad-Luc or Ad-hIRAP were injected into contralateral knees (group 2). A third set of animals (group 3) received TV or PBS in contralateral knees to challenge the efficacy of TV in the absence of vector control. A final set of animals (group 4) did not receive injections in either knee to allow reference of the above treatment groups to untreated controls. The percentage of chondrocytes staining positive for IL-1β were assigned a score of *0–5* and a scale of *1–3* was used to gauge the intensity of immunostaining (total ranging from *0–8*). Histological grading of serial coronal sections of the medial weight-bearing compartment of each knee was performed using adapted Mankin criteria based upon characteristic features of OA in this species. The score for each structure ranged from *0* (normal) to *14* (severe structural damage and complete loss of toluidine blue staining), providing a total chondropathy index ranging from *0–28*. Data were analyzed using the ANOVA, Kruskal–Wallis test, followed by Dunn's multiple comparison post-hoc test. (Please see Fig. 1 for full description of abbreviations. †$P < 0.05$.)

detected, onset of chondropathy was detected after 120 days of treatment. Additional work would be required to determine if these treatments would alter the progression and/or severity of OA in this animal model at later dates. Of note, quantification of hIRAP, type II collagen, and aggrecan, as well as IL-1β signaling molecules and additional cytokine isoforms, in the entire joint organ would provide greater insight into global responses to the chosen therapies. As sequencing and annotation of the guinea pig genome is still underway, full-scale investigations remain limited without an exhaustive list of available reagents. As such, it is plausible that compensatory or supplanting biomechanical/molecular factors not targeted in our work could have contributed to initial pathogenesis. Residual IL-1β activity may have persisted from tissues other than the examined cartilage and compensatory cytokines, including IL-1α, could have superseded therapeutic advantages. In addition, work has demonstrated persistent and robust reporter gene expression *via* self-complementary (sc)AAV[20], It is possible this vector could achieve earlier gene expression and greater effectiveness in the guinea pig model than single-stranded AAV; indeed, scAAV vector encoding hIRAP has been validated for intra-articular use[32]. Further, it may be necessary to combine interventional therapies with IL-1β reduction to achieve a more effective suppression of disease. Attempts to alter the effective concentration of IL-1β may be co-dependent on additional management – such as reduction of loading – at critical time points during disease development. Continued, and longer term, *in vivo* work is warranted to investigate therapeutic modifications that would compliment anti-IL-1β molecular therapy. Assessment of gait improvements and/or reduction in joint pain may also detect positive outcomes related to the suppression of inflammatory mediators[4]. Our findings reinforce the credence that timely identification of individuals at premature risk for OA, followed by immediate multi-modal treatment, is necessary to provide a setting that is conducive to disease suppression.

One unique finding was that IL-1β transcript and protein expression was reduced at 180 days in all animals that received either anti-IL-1β vector, including untreated knees, when compared to untreated group 4 animals. Interestingly, this is not a new or unexpected observation. Beneficial alterations in untreated contralateral limbs or regional joints has been demonstrated under a number of experimental conditions[33–36], including animal models of rheumatoid arthritis[37,38]. Two explanations for this include translocation of vector to opposite limbs or migration of transduced or immune-modulated cells to outside the joint. In particular, migration of immunotolerant dendritic cells stimulated by anti-IL-1β therapy has been described[37] and additional uncharacterized mechanisms of local or systemic cytokine suppression may also exist. Given the positive feedback loop that this pro-inflammatory molecule exerts on itself, as well as the synergism it has with other cytokines, it is also possible that even slight reduction/blockade of IL-1β may have far-reaching effects. Interestingly, diacerein is reported to have a carryover effect, providing more pain relief than placebo or non-steroidal anti-inflammatory drug (NSAID) for several weeks after treatment cessation[39]. Although additional corroboration is needed, these results could represent one of our more important findings.

Optimal viral vectors for intra-articular gene delivery for RNAi or protein production must be capable of efficient and sustained transgene expression within a reasonable vector particle dose. Our findings indicated that both AAV and Ad vectors are viable tools capable of these aims. Specifically, our results verified that AAV5 transduced weight-bearing and non-weight-bearing cartilage, which is consistent with previous work[20,40], and are effective vehicles to induce RNAi machinery *in situ*[41,42]. Importantly, CMV copy numbers and GFP transcript expression were not significantly different between 120 and 180 days, indicating persistent expression over multiple months. As expected, evidence of transduction of articular cartilage by Ad was not demonstrated; however, luciferase expression from other articular structures was confirmed for at least 120 days in our study, which is longer than the 2–3 week period of transgene expression typically purported for Ad in most tissues of interest[43].

Adverse cellular responses must be nominal in the presence of viral vectors and these delivery vehicles must not detrimentally influence or unseat the intended therapy. AAV vectors have been associated with minimal toxicity and immune responses[43–45]. In the current work, TNF-α, IL-8, and IFN-γ were statistically increased by TV relative to paired PBS-treated knees 60 days after injection. These relative increases in TNF-α and IL-8 expression levels induced by AAV vectors, however, appeared to be transitory[43] and were statistically decreased 120 days post-injection. A persistent increase in IFN-γ was present in both TV and NTV knees, which is most likely related to vector use and not RNAi mechanisms. Our results support other peer-reviewed studies, which demonstrated cell-mediated immune responses to AAV capsid antigens[46,47] and persistent, serotype-specific IFN-γ responses. Further, *in vitro* IFN-γ release assays have shown differences in species susceptibility to immunogens or mitogens, emphasizing the importance to scrutinize immune responses to AAV vectors in the context of individual animal models[48] and tissue types. Continued work is necessary to examine the influence of innate and adaptive immune responses to AAV5 vectors on our results in the guinea pig, with emphasis on the significance of IFN-γ in cartilage.

Ad vector particles elicit strong immune responses, often in a dose-dependent manner, and systemic delivery has resulted in rapid physiological responses that include activation of intrinsic defense mechanisms, induction of inflammation, transient liver toxicity, and thrombocytopenia[49]. These vectors activate innate immunity through toll-like receptor (TLR)-dependent and TLR-independent pathways, causing an upregulation of type I IFNs and inflammatory cytokines[50]. Adaptive responses against Ad may be directed against the capsid, double-stranded DNA genome, viral proteins expressed from the vector backbone, or incorporated transgenes[43]. In comparison to PBS-injected knees, Ad vector-exposed cartilage had a significant increase in inflammatory cytokine expression, specifically TNF-α, IL-8, and IFN-γ, especially at 120 days. Given the adaptive immune response anticipated in response to Ad, as well as the reversal of the potentially beneficial alterations in TGF-β and MMP13 expression between 120 and 180 days, it would be interesting to determine if there was evidence at the latter time point of host response to foreign antigens. Additional studies are needed to investigate immune responses specific to the joint environment and to ascertain if viral vector use usurped or prejudiced the beneficial effects of the investigated therapies.

There are theoretical advantages to using RNAi vs standard gene delivery of a therapeutic product. First, as selected shRNA sequences are reliably and constitutively transcribed from polymerase III promoters and efficiently processed by the RNA-induced silencing complex, RNAi does not face hurdles related to proper protein production. Second, the development of techniques to deliver long-lasting siRNAs to cells in the absence of a delivery vehicle or viral vector may eliminate negative consequences associated with vector/DNA administration. Finally, although it is unknown the extent to which dsRNA stimulates induction of innate cellular immune responses, this obstacle may be easier to overcome than conventional gene delivery due to the ability to identify highly potent RNAi sequences. Efficient small interfering RNA molecules decrease the likelihood of off-target effects, decrease the concentration of dsRNA molecules required, and may be better able to evade host responses. In the context of the current study,

chondrocytes may also have different adaptive responses to a reduced IL-1β signaling at the transcript level compared to receptor antagonism of the IL-1 type I receptor by hIRAP. Further effort is required to determine which method pursued in this study may be more effective.

In summary, this work demonstrated that a reduction in IL-1β signaling by shRNA-mediated RNAi or receptor blockade by hIRAP was achieved in the Hartley guinea pig model of OA. Importantly, this manipulation was demonstrated over a 60-day period. Reducing the effective concentration of IL-1β decreased expression of three inflammatory mediators and one catabolic agent, while simultaneously increasing the number of anabolic TGF-β transcripts, providing biological evidence that IL-1β serves an important role in primary OA.

Contributions

Kelly S. Santangelo: design of the study, obtaining of funding, acquisition of the data, analysis and interpretation, drafting and revising of the article, and final approval. Responsibility for integrity of the work.

Gerard Nuovo: Acquisition of the data, analysis and interpretation, revising of the article, and final approval.

Alicia L. Bertone: design of the study, obtaining of funding, analysis and interpretation, revising of the article, and final approval. Responsibility for integrity of the work.

Conflict of interest

The authors declare that there is no conflict of interest.

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Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.joca.2012.08.011.

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